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ALABAMA UNIV IN BIRMINGHAM LAB OF MOLECULAR BIOPHYSICS
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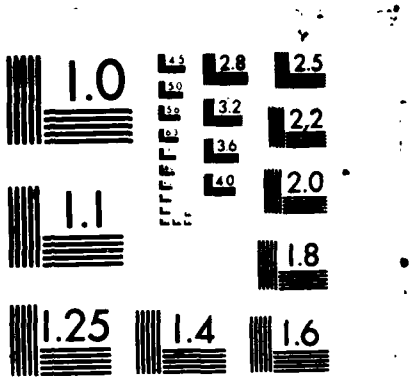

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Urry, Dan W., Hunter, Eric, Prasad, K. U., Long, Marianna M.

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Development of elastomeric polypeptide biomaterials indicate that polypeptide elastic processes may be either a passive and simple case of resistance to and recovery from a mechanical deformation or an active and coupled case of development of a motive force by transduction: thermomechanical, chemomechanical or electromechanical. In the coupled case, a transition between two states of different degree of elasticity may be achieved by a change in temperature, a change in chemical potential or a change in electrical potential. Commonly it is the situation of the temperature of a thermal transition being shifted by a change in chemical or electrical potential. Preliminary data suggest that repeating peptide sequences of novel design can provide elements of each case and that the temperature for the underlying thermal transition can be widely varied. The basic elastomeric repeating sequences are the polypentapeptide $(Val^1\text{-}Pro^2\text{-}Gly^3\text{-}Val^4\text{-}Gly^5)_n$ and the

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Dr. E. Schmell, M. Marron or M. Haygood	(202) 696 4760	ONR

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19. ABSTRACT (Continued)

polytetrapeptide $(Val^1\text{-}Pro^2\text{-}Gly^3\text{-}Gly^4)_n$, and the inelastic polyhexapeptide (Val-Ala-Pro-Gly-Val-Gly)$_n$ can be introduced to improve strength and handling characteristics of the elastomer.

The polymers that have been chemically prepared are the Ile^1-, Ile^4-, Leu^1-, Leu^4-, Phe^1-, Phe^4-, Ala^3-, 20% Glu^4- and 20% Lys^4- polypentapeptide; Ile^1- and D·Ala^3- polytetrapeptide; polyhexapeptide and polypentapeptide mixes (1:1, 1:2, 1:4, 1:8), and two-component models of polypentapeptide with sequences of n = 15 separated by two different alanine-rich, glycine-containing cross-linking sequences. In order to provide a system for the biosynthesis of wild type and altered forms of the elastin polypentamer, the possibility of expressing elastin polypentamer sequences in a microbial vector system has been investigated. For these studies, two genes, each 150 base pairs in length, have been synthesized that can encode a 10mer repeat of the basic repeat unit - Val-Pro-Gly-Val-Gly. The first of the genes utilizes all 12 available codons for the 3 amino acids whereas the second uses a more limited subset preferentially used by E. coli. These synthetic genes have been cloned into the SmaI site of the bacterial expression vector pUC118 to provide an in-frame fusion protein with the alpha-subunit of β-galactosidase. Studies to identify and characterize the products of this construction are currently ongoing.

Physical characterizations underway utilize temperature dependence of composition leading to phase diagrams, temperature profiles of aggregation, of ellipticity, of dielectric relaxation, of nuclear magnetic resonance, of elastic modulus and of force development, and differential scanning calorimetry. Special experiments are examining the coupling of extension to dielectric response, and the temperature dependence of the development of the elastomeric state as a function of pH for 20% Glu^4- polypentapeptide.

ANNUAL REPORT

Contract Title: Development of Elastomeric Polypeptide Biomaterials

Contract Number: N00014-86-K-0402

I. Brief Summary of Project Goals

To design, prepare and characterize novel elastomeric polymers comprised of repeating peptide sequences, primarily the elastin pentamer and analogs of it alone and combined with repeating related hexapeptides and/or tetrapeptides. The purpose is to develop polymers with different elastic moduli and increased extension limits, polymers with different temperature ranges for their inverse temperature transitions over which elastomeric force dramatically changes, polymers in which different heat changes effect the large changes in elastomeric force, polymers with different intensities and frequencies of their dielectric relaxations and polymers with wider temperature ranges over which they function as nearly ideal elastomers. In the elastomer design, the dominant repeat units will be pentamers and tetramers. Hexamers and alanine-rich, lysine-containing cross-linking sequences will be used to fine-tune properties.

II. Summary of Accomplishments in the First Year

The research efforts naturally divide into two major parts: A. syntheses, chemical and microbial, and B. physical characterizations. In what follows will be a brief statement of the chemical synthesis and a longer statement of the microbial synthesis as no manuscript has yet been written up in this area, and for the physical characterizations will be attached several manuscripts: five are in press (three of which are invited manuscripts) and the sixth has been submitted for publication. The three invited manuscripts represent development, generalizations and extensions of the concepts derived from the work on elastomeric polypeptide biomaterials.

A. Synthesis

1. Chemical Synthesis

Polypentapeptides: The syntheses of polymeric peptides were carried out for the most part by classical solution methods. Polymerization was carried out both by the C-terminal Gly-activation and by the C-terminal Pro-activation. That is, when Ile^1-PPP was synthesized, the starting monomers were H-IPGVG-ONP and H-GVGIP-ONP. Similarly, other polymers, Ile^4-, Leu^1-, Leu^4-, Phe^1-, Phe^4-, D·Ala^3-polypentapeptides were synthesized. For simplicity, either a 2+3 or a 3+2 approach was used. For example, in the case of Ile^1-pentamer, Boc-GVG-OBzl(I) and Boc-IP-OBzl(II) were first synthesized by the mixed anhydride method. In one instance, II was hydrogenated, I was deblocked and both were coupled using EDCI to obtain Boc-IPGVG-OBzl, i.e., the pentamer for Gly activation. In the second case, I was hydrogenated, II was deblocked and both were coupled to give Boc-GVGIP-OBzl which is used for Pro activation. After hydrogenating the pentapeptide, it was converted to the p-nitrophenyl ester using bis(p-nitrophenyl) carbonate, deblocked and polymerized. A similar procedure was used in other polymer preparations also.

In the case of Glu and Lys containing polymers, the pentapeptide Boc-VPGE (Bu^t)G-ONp and Boc-VPGK(Z)G-ONp were prepared and mixed with Boc-VPGVG-ONp in 1:4 ratios, deblocked and copolymerized to obtain 20% Glu^4- and 20% Lys^4-containing polypentapeptides. The list of polypentapeptide analogs that have been synthesized are given in Table 1 along with the temperature for the onset of coacervation.

Polytetrapeptides: Ile^1-PTP was prepared starting from the monomer Boc-GGIP-ONp and D·Ala^3-PTP was prepared from Boc-D·Ala GVP-ONp. The monomers themselves were prepared by the mixed anhydride method.

Two Component Models: Two component models of elastin were synthesized by a combination of solution and solid phase methodology. In the

solid phase method, both stepwise condensation and segment coupling approach were used. The first pentamer unit Boc-VPGVG-resin was built onto the resin by the stepwise condensation method. The next 14 pentamer units were attached by the segment coupling technique using Boc-VPGVG-OH in the presence of DCC-HOBt. After all the 15 pentamer units were attached to the resin, the two cross-linking sequences AAAAKAAKYGA (XL-1) and AAKAAAKAA (XL-2) were then added by the stepwise condensation method. The peptides were then removed from the resin as methyl esters by transesterification with CH_3OH/DMF-Et_3N. After purifying each peptide by repeated precipitations from a mixture of different solvent systems, it was saponified and converted to p-nitrophenyl ester. The N-terminal blocking group was removed and the peptide active ester was polymerized for several days. The polymeric solution was diluted with water, dialyzed against water using a 50kD molecular weight cut-off dialysis tubing and lyophilized. Thus, Poly[XL-1-$(VPGVG)_{15}$]-OH f and Poly[XL-2-$(VPGVG)_{15}$]-OH, were obtained with molecular weights of greater than 50,000 daltons.

The intermediate and final products were all characterized as appropriate by thin layer chromatography, C-13 magnetic resonance spectra and amino acid analysis. In Table 2 are listed the polytetrapeptide analogs that have been synthesized as well as the two component models.

2. Microbial Synthesis of Elastin Polymers

Recent advances in oligonucleotide synthesis and molecular cloning technologies provide new opportunities for the design and high level expression of a variety of novel gene products. The initial approaches we have proposed in order to investigate the possibility of expressing elastin polymers in microbial cells were: 1) To synthesize synthetic genes that encode the basic polypentamer repeat unit; 2) Insert these synthetic genes into the bacterial expression plasmid pUC118; 3) Express the elastin sequences as a fusion polypeptide with the alpha-subunit of β-galactosidase and 4) Investigate procedures for

purification of the fusion protein. We have made significant progress towards achieving these goals during the current funding period.

a. Synthesis of polypentamer-encoding genes: In order to generate a double stranded DNA fragment of 150bp capable of coding for a decameric repeat of the basic (Val-Pro-Gly-Val-Pro) pentamer, we have synthesized overlapping oligonucleotides that after hybridization and enzymatic extension yield the 150bp synthetic gene. In addition to the two oligonucleotides described in the initial proposal (JG1 and JG2, shown below) which were designed to yield the lowest redundancy based on codon redundancy, we have synthesized two additional oligonucldotides in which we have optimized the sequence for prokaryotic codon usage according to Ikemura and Ozeki (1982). The respective oligonucleotides are shown below.

JG1-82mer
5'-GTTCCTGGTGTTGGTGTCCCCGGCGTCGGCGTACCAGGAGTAGGAGTGCCGGGGGTGGGGGTTCCCGGAGTGGGTG
TCCCAGGG-3'

JG2-85mer
5-TCCGACACCGGGTACCCCTACGCCAGGGACACCAAACGCCTGGCACACCGACTCCCGGTACGCCTACCCCTGGGACA
CCCACTCCG-3'

JG3-85mer
5'-GTTCCGGGTGTTGGTGTACCGGGTGTTGGTGTGCCGGGTGTTGGTGTTCCGGGCGTAGGCGTACCGGGCGTAGGCG
TGCCGGGCG-3'

JG4-85mer
5'-ACCTACACCCGGAACGCCCACACCCGGCACGCCCACGCCCGGTTACGCCCACGCCCGGAACGCCTACGCCCGGCAC
GCCTACGCCC-3'

All four olignucleotides were synthesized in the UAB oligonucleotide core facility on an Applied Biosystems oligonucleotide synthesizer. The higher GC content of these oligonucleotides significantly lowered the efficiency of coupling and thus the overall yield of the product. The overall yield of full length oligonucleotide calculated after end labelling with gamma-labeled ATP and electrophoresis on a 6% polyacrylamide gel was approximately 5.0% (versus >50% seen with shorter, less GC rich oligonucleotides). Each oligonucleotide was purified from a preparative polyacrylamide gel and the actual yield in µg calculated from

the O.D. 260 of the solution.

Approximately 300ng of oligonucleotides JG1 and JG2 or JG3 and JG4 were annealed and the 3' ends extended with either the Klenow fragment of E. coli DNA polymerase I or with AMV reverse transcriptase, to generate double stranded DNA fragments of approximately 150bp. The efficiency of double strand synthesis was significantly better when reverse transcriptase was used for this reaction; probably reflecting its ability of meltout G-C rich hairpins. The products of the reaction, which contained trace levels of ^{32}P-dATP were separatd on preparative 4% polyacrylamide gels (Figure 1), together with appropriate size markers, the region containing the 150bp band excised and the fragment eluted. Approximately 0.6µg of double-stranded product was obtained in each case.

b. Cloning into the expression vector: The initial vector of choice for these studies is pUC118 (Messing and Viera, 1985). This cloning/expression vector contains a polylinker cloning site in the β-galactosidase coding region; expression being under control of the lacZ operator and promoter. Cloning of the 150bp synthetic gene into the unique SmaI site in this vector results in an in-frame gene fusion and a hybrid polypeptide that consists of a decameric repeat of the elastin pentamer and the first 80 amino acids of β-galactosidase (α-subunit). Since this fusion protein retains the ability to complement bacterial strains with a defective β-galactosidase α-subunit, bacteria carrying the recombinant pUC118 yield blue colonies in the presence of X-gal and the inducer IPTG.

In initial experiments, we attempted to clone directly into the SmaI cut pUC118 plasmid, and following transformation to screen colonies by colony hybridization using ^{32}P-labelled oligonucleotides. The higher G+C content of the oligonucleotides however resulted in very high background and an inability to identify positive cloning. We have therefore first cloned into

the SmaI site of the single stranded phage M13mp18. The cloning of tanden arrays of the insert was avoided by leaving the double stranded 150 base pair insert non-kinasee (i.e., without 5'-phosphate). Those phage containing the insert in the correct orientation for expression could be identified by using only JG1 and JG3 oligonucleotides to probe nitrocellulose lifts of the ensuing phage plaques. A typical series of plaque lifts is shown in Figure 2.

Approximately 20 plaques for each of the JG1/JG2 and JG3/JG4 series were picked and both double stranded RF form DNA and single stranded phage DNA was prepared. The insert in each was sized by cutting the RF DNA with EcoRi and PstI (unique enzyme sites that flank the insert; Figures 3 and 4) and by sequencing using the M13 universal primer. Many of the inserts which yielded blue plaques (i.e., in-frame fusions) nevertheless represented incomplete copies of the synthetic gene. For each of the 2 synthetic genes, one clone, containing a full length in-frame copy of the gene, was chosen, and the 190 base pair EcoRI/PstI fragment transferred to pUC118. The presence of the fragment was confirmed by restriction enzyme analysis and the insert sequenced by the Maxam-Gilbert chemical cleavage method. In this way, we have confirmed the sequence and in-frame fusion for both the JG1/JG2 and JG3/JG4 genes. The sequencing studies did reveal two nucleotide changes in the JG1/JG2 gene (one A to C and one C to A) but neither affected the amino acid encoded by the codon.

c. Expression of the Polypentamer Sequences: Since bacteria containing the pUC118 plasmids with either full length insert yielded blue colonies in the presence of IPTG and X-gal, it is clear that both fusion proteins can be synthesized in this prokaryotic system. Furthermore the inserts, despite their redundancy of sequence, appear to be maintained in a stable fashion in their bacterial hosts in the absence of induction. The expected size of the fusion product is in the order of 13-14kD and we have initiated experiments to

identify and characterize the product synthesized in induced cells. In these preliminary experiments, we have grown cells overnight in minimal medium containing glucose, diluted 1:100 in B-broth and grown to an OD 600 of 0.6 prior to inducing the lac-fusion gene expression with IPTG. Initial results indicate the appearance, in cells containing the JG3/JG4 gene, of a novel protein doublet of the expected molecular weight for the fusion protein. Additional experiments will be required to confirm the identity of these inducible products.

B. Physical Characterizations

While there are numerous physical characterizations that are in various stages of completion, included in the present report are the following six manuscripts:

1. D. W. Urry, "Entropic Elastomeric Force in Protein Structure/Function," Int. J. Quantum Chem.: Quantum Biol. Symp., 14 (in press).

2. K. U. Prasad, M. Iqbal and D. W. Urry, "Synthesis of Two Component Models of Elastin," Proceedings of the Tenth American Peptide Symposium, 1987 (in press).

3. D. W. Urry, "Entropic Elastic Processes In Protein Mechanisms. Part 1. Elastic Structure Due To An Inverse Temperature Transition and Elasticity due to Internal Chain Dynamics," J. Protein Chem. (in press).

4. D. W. Urry, "Entropic Elastic Processes in Protein Mechanisms. Part 2. Simple (Passive) and Coupled (Active) Developments of Elastic Forces," J. Protein Chem. (in press).

5. D. W. Urry, "Elastic Molecular Machines and a Motive Force in Protein Mechanisms," Proceedings of the Materials Biotechnology Symposium. Natick Research, Development and Engineering Center and the U. S. Army Research Office (in press).

6. R. Buchet, C.-H. Luan, K. U. Prasad, R. D. Harris and D. W. Urry, "Dielectric Relaxation Studies on Analogs of the Polypentapeptide of Elastin," J. Phys. Chem. (in press).

TABLE 1

CHEMICAL SYNTHESES

Polypentapeptide (PPP) Analogs

		Transition Temperature (TP_T)
PPP	$(Val^1\text{-}Pro^2\text{-}Gly^3\text{-}Val^4\text{-}Gly^5)_n > 120$	25°C
Ile^1-PPP	$(Ile^1\text{-}Pro^2\text{-}Gly^3\text{-}Val^4\text{-}Gly^5)_n > 120$	9°C
Ile^4-PPP	$(Val^1\text{-}Pro^2\text{-}Gly^3\text{-}Ile^4\text{-}Gly^5)_n > 120$	12°C
Leu^1-PPP	$(Leu^1\text{-}Pro^2\text{-}Gly^3\text{-}Val^4\text{-}Gly^5)_n > 120$	14°C
Leu^4-PPP	$(Val^1\text{-}Pro^2\text{-}Gly^3\text{-}Leu^4\text{-}Gly^5)_n > 120$	15°C
Ala^3-PPP	$(Val^1\text{-}Pro^2\text{-}Ala^3\text{-}Val^4\text{-}Gly^5)_n > 120$	34°C
Phe^1-PPP	$(Phe^1\text{-}Pro^2\text{-}Gly^3\text{-}Val^4\text{-}Gly^5)_n$ = ?	< 0°C
Phe^4-PPP	$(Val^1\text{-}Pro^2\text{-}Gly^3\text{-}Phe^4\text{-}Gly^5)_n$ = ?	< 0°C
50% Phe^1-PPP	$(Xxx\text{-}Pro^2\text{-}Gly^3\text{-}Val^4\text{-}Gly^5)_n$; Xxx = Phe^1:Val^1; 1:1	
50% Phe^4-PPP	$(Val^1\text{-}Pro^2\text{-}Gly^3\text{-}Xxx\text{-}Gly^5)_n$; Xxx = Phe^4:Val^4; 1:1	
20% Glu^4 PPP	$(Val^1\text{-}Pro^2\text{-}Gly^3\text{-}Xxx\text{-}Gly^5)_n$; Xxx = Glu^4:Val^4; 1:4	
	pH = 2;	39°C
	pH = 7;	52°C
20% Lys^4-PPP	$(Val^1\text{-}Pro^2\text{-}Gly^3\text{-}Xxx\text{-}Gly^5)_n$; Xxx = Lys^4:Val^4; 1:4	
	pH = 9	70°C
	pH = 11;	55°C

TABLE 2

CHEMICAL SYNTHESES

Polytetrapeptide (PTP) Analogs

		Transition Temperature (TP_T)
PTP	$(Val^1\text{-}Pro^2\text{-}Gly^3\text{-}Gly^4)_n > 120$	49°C
Ile^1-PTP	$(Ile^1\text{-}Pro^2\text{-}Gly^3Gly^4)_n > 120$	20°C
D•Ala^3-PTP	$(Val^1\text{-}Pro^2\text{-}D\text{•}Ala^3\text{-}Gly^4)_n > 120$	

Two Component Models

Poly[XL-1-$(VPGVG)_{15}$]; XL-1 = AAAAKAAKYGA

Poly[XL-2-$(VPGVG)_{15}$]; XL-2 = AAKAAAKAA

Polypentapeptide : Polyhexapeptide Mixes

1:1, 1:2, 1:4, 1:8

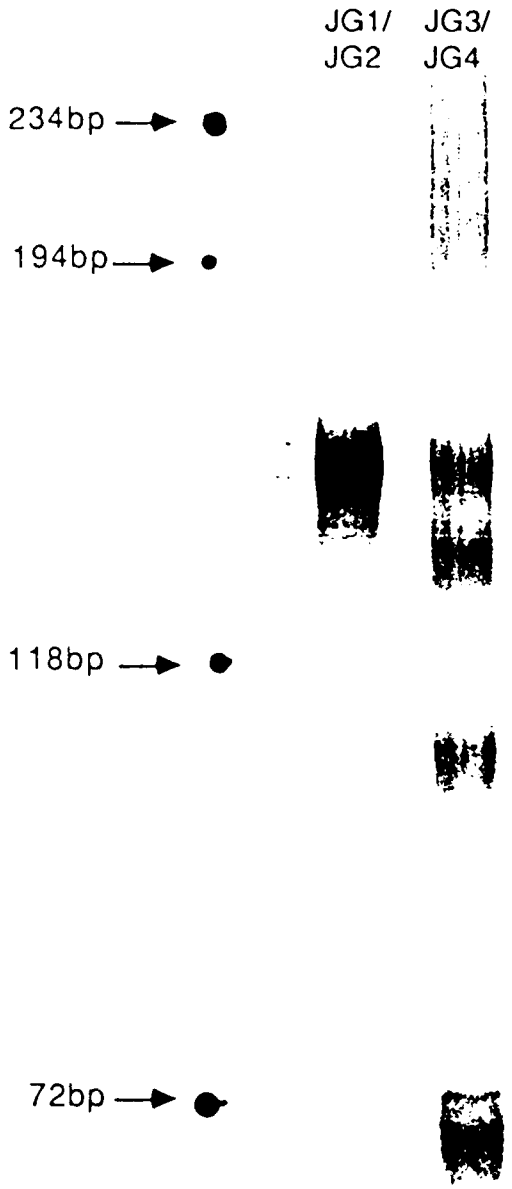

Figure 1. Synthesis of double stranded polypentamer gene. Oligonucleotides JG1 and JG2 or JG3 and JG4 were annealed then extended with Reverse transcriptase and all 4 dNTPs. Incomplete double stranded fragments in JG3/JG4 lane may represent the products of hair-pin priming.

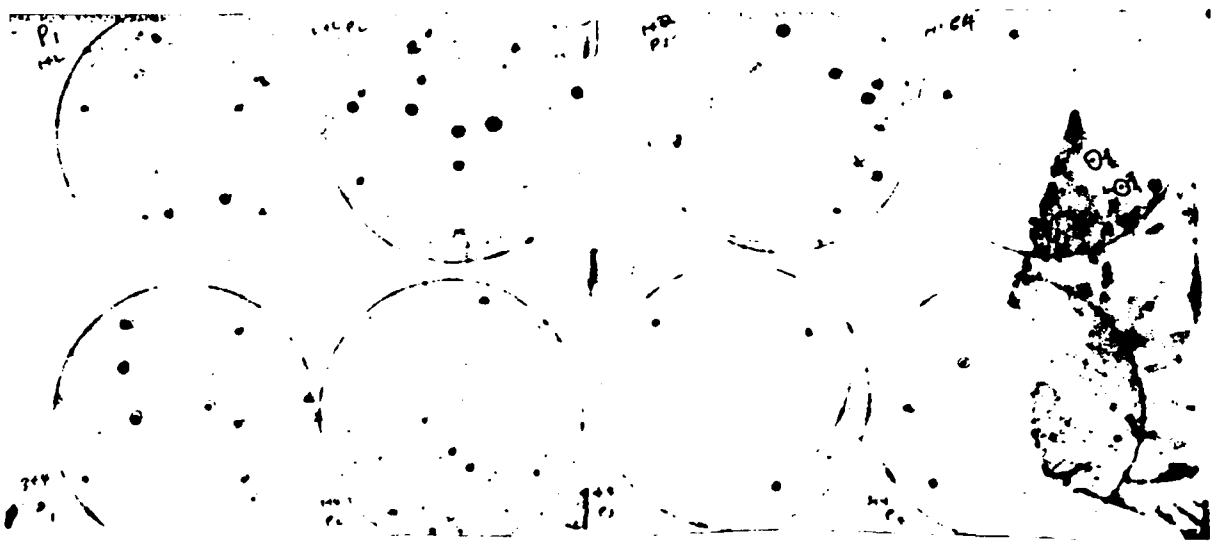

Figure 2: Plaque hybridization with JG1 and JG3 oligonucleotides: Phage plaques were transferred to nitrocellulose and hybridized at moderately high stringency to end-labelled oligonucleotides. Positive plaques were identified on the plate, picked and phage grown for sequence studies.

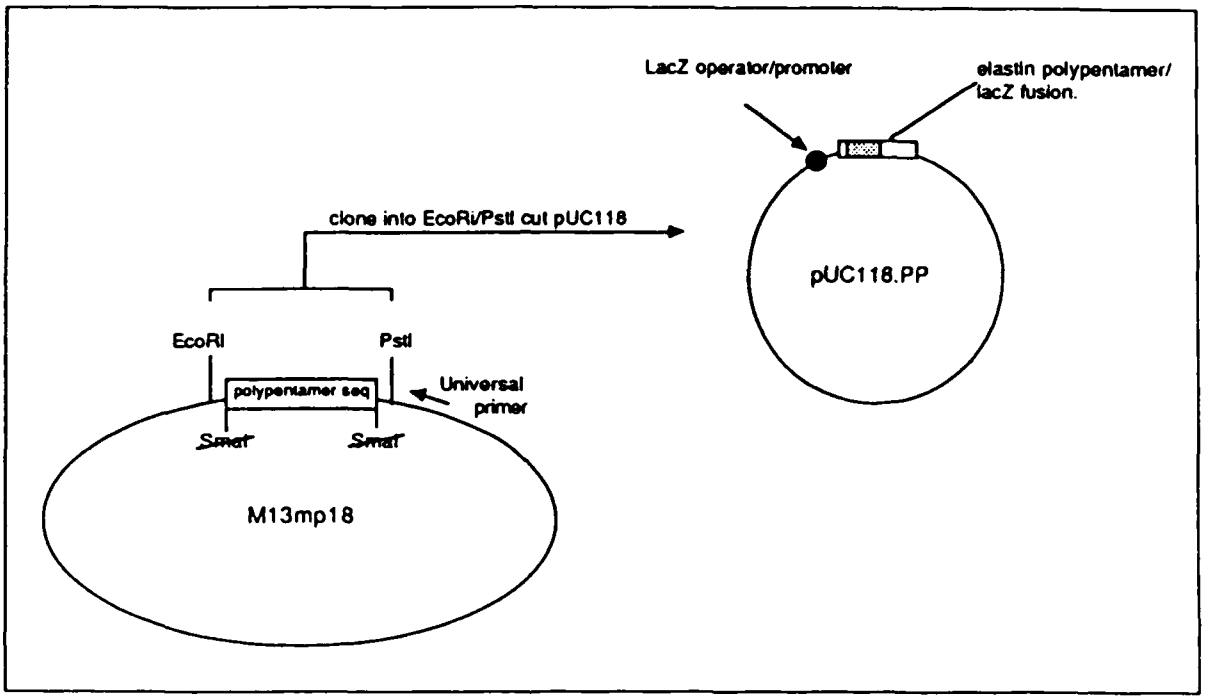

Figure 3: Isolation and subcloning of synthetic polypentamer gene: Cloning of the double stranded polypentamer gene sequence into the SmaI site of M13mp18 destroys the site. The gene can be transferred to pUC118 by digestion with EcoRI and PstI to generate an in-frame fusion protein

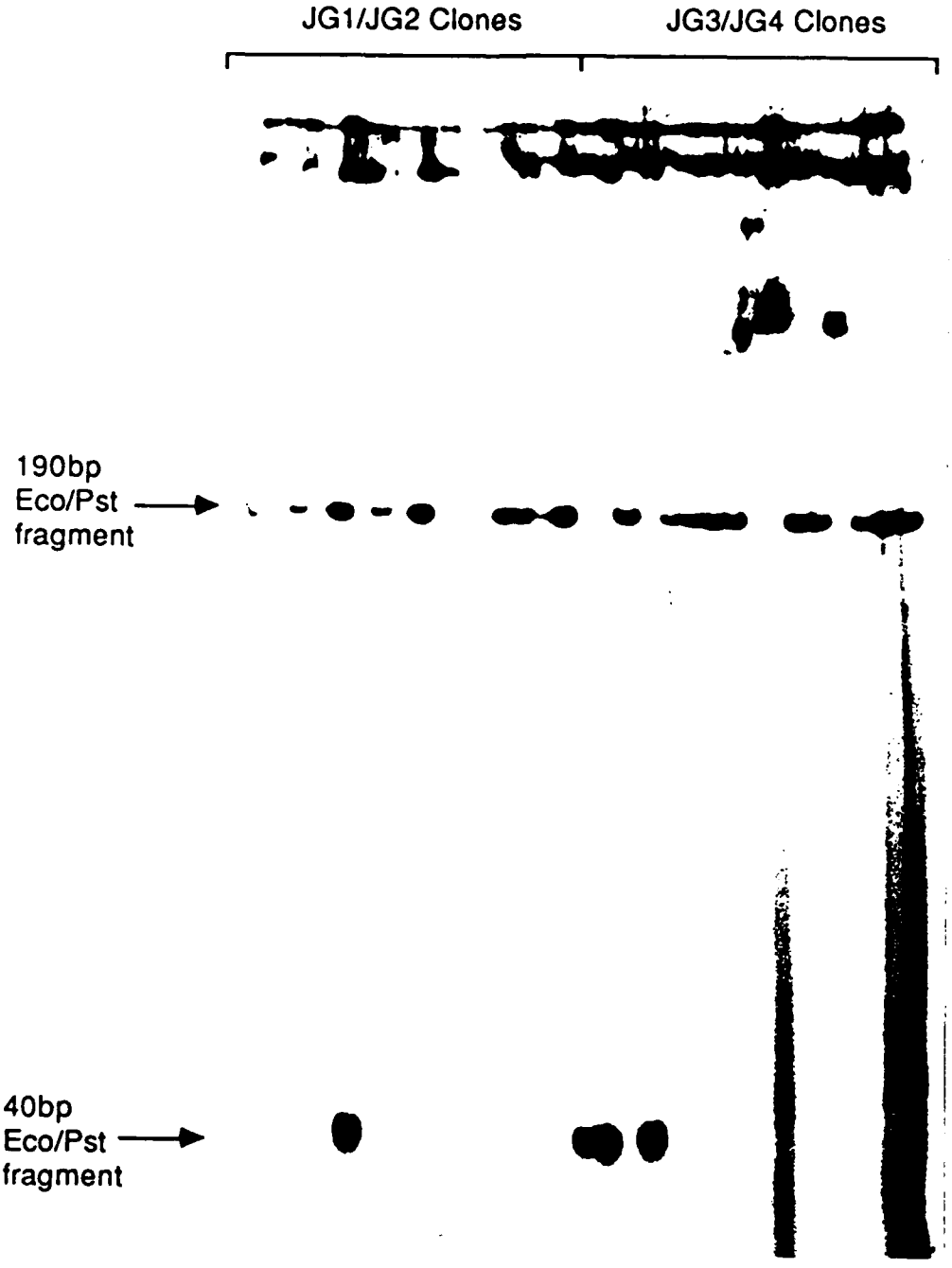

Figure 4: Phage RF DNA was digested with EcoRI, blunt-ended with Klenow and P-labelled dNTPs then digested with PstI. Labelled fragments were separated by 4% PAGE. The 40bp fragment is derived from phage lacking an insert.

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